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14. ABSTRACT
There is accumulating evidence suggesting that survivin, a member of the inhibitor of apoptosis (IAP) family, is associated with both progression of prostate carcinoma and drug resistance. Therefore, we hypothesized that survivin plays a role in the development of hormone-refractory prostate cancer (HRPC) and resists killing by chemotherapeutic agents; thus the down-regulation of survivin by DIM, a non-toxic dietary compound formed in the stomach after consumption of Brassica vegetables like broccoli or cabbage, has been known to have cancer chemopreventive activity with greater bioavailability could enhance killing of hormone insensitive prostate cancer cells by Taxotere. Recently, we discovered that DIM-induced apoptosis is associated with down-regulation of survivin in breast cancer cells. We also found that DIM significantly sensitized the breast cancer cells to Taxotere-induced killing. Therefore, we would like to test whether a similar pathway is playing a role in prostate cancer, especially HRPC and bone metastatic disease. To test our hypothesis, we will investigate whether treatment of prostate cancer cells (LNCaP, AR +, responsive to androgen and C4-2B, AR +, non-responsive to androgen) in vitro and in vivo with DIM alone or in combination with Taxotere could show greater anti-tumor effects and whether this effect is mechanistically associated with inactivation of survivin signaling. To test our hypothesis, we will employ several techniques such as MTT, ELISA, Western blot, EMSA, cDNA and siRNA transfection including an animal study. The data obtained from our experiments will provide new mechanistic insight for discovering novel approaches for the treatment of HRPC and bone metastatic disease in the future.

15. SUBJECT TERMS
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Introduction

Prostate cancer is one of the leading causes of death among men in the United States (1-3). Almost all prostate cancer patients treated with hormone ablation therapy develop hormone refractory prostate cancer (HRPC) and bone metastatic disease with functional androgen receptor (AR) (1, 4, 5). Progression of prostate cancer to androgen independence remains the primary barrier to improving patient survival due to complex mechanisms underlying the evolution to androgen independence, and it remain poorly understood (6). Moreover, effective treatment for men with hormone-refractory prostate cancer (HRPC) has not yet very successful. Therefore, it is an important challenge to develop effective strategies for the treatment of HRPC and bone metastasis. Moreover, understanding the molecular mechanisms of progression of prostate cancer to HRPC is the first step toward the development of specific therapeutic strategies (6).

It has been well known that many genes play important roles in the control of cell growth, differentiation, apoptosis, inflammation, stress response and many other physiological processes (7-19). It is known that apoptosis has a central role in the development of prostate cancer and its progression to an androgen-independent state which is, in part, due to up-regulation of anti-apoptotic genes after androgen deprivation (1, 10, 15, 20-28). Several studies have suggested that survivin, a new member of the inhibitor of apoptosis (IAP) family, plays important roles in tumorigenesis, progression of breast carcinoma, cell invasion, metastasis and resistance to chemotherapy and cancer cell survival in many types of cancer, including HRPC (1, 17-19, 29-42). Survivin is also highly articulated in all major tumor types (43) but is undetectable in most normal differentiated tissues. Because a correlation exists between high expression of survivin in tumors and poor survival among patients with various cancers (44-46). Therefore, survivin is considered a novel target in various cancer therapies. In this study we examined whether survivin could play a potentially important role in HRPC and bone metastatic disease and that targeting of the survivin pathway could enhance therapeutic efficacy.

There is accumulating evidence suggesting that survivin, is associated with both progression of HRPC and drug resistance (1, 28-35). Therefore, suppressing survivin may be a novel and effective therapeutic approach for HRPC. However, very little or no information is currently available regarding the consequence of blocking survivin signaling, which may lower the anti-apoptotic threshold in cancer cells, thus sensitizing prostate tumor cells to apoptosis. Several studies on survivin have revealed that a number of existing anticancer drugs show survivin-suppressive

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activity through various cell signaling pathways (17-19, 47-52). But the safety and toxic profile are though to important for their efficacy in cancer chemotherapy. Thus, there is a dire need for the development of a novel and specific anti-survivin therapeutic strategy for the treatment of HRPC.

Previous studies have shown that DIM, a major *in vivo* acid-catalyzed condensation product of I3C, is thought to have a protective effect against the development of human prostate cancer including breast cancer; however their function in HRPC therapy is not known (10, 11, 14, 15, 24, 25, 53-59). It has also been indicated that HRPC is an aggressive and treatment-resistant disease and the pharmacological therapeutic approach for this disease has previously shown limited efficacy (60-62). In spite of the obvious importance of DIM as a cancer chemopreventive agent, knowledge on the molecular mechanism(s) of action of DIM against prostate cancer and their role in cancer therapy is lacking. Several molecular mechanisms that could be related to the multiple gene alterations observed in advanced prostate cancer are also involved in the transition towards an androgen-independent stage, including changes in cell growth and anti-apoptotic factors such as Akt/NF-κB and survivin (1, 10, 15, 20-24, 26, 27). It has been reported that over-expression of survivin is associated with the progression of Prostate cancer (1, 28, 34, 63, 64) and it seems to be a important target for the treatment of HRPC. In HRPC, function of AR, together with the activation of Akt/NF-κB pathways, also promotes cancer cells to become resistant to androgen deprivation therapy (65-68) In addition, previous studies have shown that androgen could induce oxidative stress resulting in the production of reactive oxygen species, which in turn could activate NF-κB and contribute to the induction of cell growth during the development or progression of prostate cancer (69-72). However, the down regulation of survivin via inactivation of inactivation of AR and NF-κB signaling by DIM could be an important strategy for the treatment of prostate cancer, especially HRPC. In this study, we examined whether DIM increases apoptotic cells death thereby inactivating survivin leading to chemosensitization of HRPC cells to Taxotere-induced killing using both androgen responsive LNCaP cells (AR positive) as well as androgen independent C4-2B cells (AR positive) which is more similar to human HRPC. Here we report the mechanistic role of a non-toxic dietary agent such as DIM for the treatment as well as enhancement of the therapeutic efficacy of Taxotere for prostate cancer in general but most importantly for HRPC and bone metastatic disease. We believe that targeting survivin by DIM could be a novel approach for the treatment of HRPC and bone metastatic disease.

Body Report

The original statement of work in the proposal is listed below:

Task 1: (Months 1-10)

Aim 1: **To study whether treatment of prostate cancer cells with DIM could inactivate survivin signaling associated with the expression of other genes leading to apoptotic cell death.** To test this hypothesis, we will determine whether treatment of prostate cancer cells [androgen sensitive (LNCaP, AR+) and androgen insensitive (LNCaP derived C4-2B, AR+)] with DIM could inactivate survivin signaling, thereby inactivating AR and NF-κB signaling.

(1) We will conduct experiments in four groups such as control untreated, DIM (10 μM), Taxotere (1.5nM) and DIM plus Taxotere using both LNCaP as well as C4-2B cells in culture using 96-well plate MTT assay following standard procedure described by our laboratory previously. We will determine whether over-expression by several gene transfections could abrogate DIM-induced prostate cancer cell death. Therefore, studies will be conducted in cell extracts prepared from cells exposed to DIM/Taxotere +/-AR cDNA (OriGene Technologies, Inc. MD), p65cDNA and survivin cDNA (Science Reagent, USA) transfections for measuring cell growth inhibition by MTT assay and for the measurement of apoptotic cell death by ELISA as described previously by our laboratory. The experiment will be repeated three times and statistical data analysis will be performed to verify the significance of cell growth inhibition after specific treatments. We will measure NF-κB DNA-binding activity, protein levels of AR and survivin in prostate cancer cells in the presence and absence of DIM +/- Taxotere by EMSA and Western blot analysis, respectively, as described previously by our laboratory. We will also conduct apoptosis assays (ELISA, PARP, caspase activation) under all experimental conditions and correlate apoptosis data with NF-κB and survivin data. These experiments will provide the information as to whether DIM, which inactivates AR, NF-κB and survivin in prostate cancer cells, could lead to apoptotic cell death and cause chemo-sensitization of prostate cancer cells to Taxotere.

(2) In addition, we will test whether the down-regulation of survivin by siRNA approach may confer better and enhances sensitivity of prostate cancer cells to DIM-induced cell growth inhibition

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and apoptosis measured by standard procedures. siRNA will likely be purchased from commercial sources (Upstate, Waltham, MA), and we will use Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for transfection. We have established siRNA transfection technique in our laboratory and these experiments will be performed using both LNCaP and C4-2B cells transiently transfected with survivin siRNA, as indicated above. Subsequently, cultured cells will be treated in the presence or absence of DIM +/- Taxotere to determine whether survivin pathways play any direct/indirect role in prostate cancer cells in conjunction with regulation of AR and NF-κB. All experimental end points will be evaluated by standard apoptosis assays as described above.

(3) A recent study showed that the promoter region of survivin has several NF-κB binding sites upstream of pLuc-649 and downstream of pLuc-1430. Therefore, we will also study whether the survivin-Luc construct and NF-κB-Luc activity could be inhibited by DIM treatment in LNCaP and C4-2B cancer cells transfected with the survivin-Luc construct and NF-κB-Luc constructs, respectively. This will provide direct evidence in support of our hypothesis that DIM down-regulates survivin through NF-κB mediated transcriptional inactivation, thereby inactivating AR, leading to apoptotic cell death and chemosensitization of HRPC to Taxotere.

Task 2: Months 10-18.

Aim 2: **To investigate whether DIM could down-regulate survivin expression both *in vitro* and *in vivo*, leading to chemo-sensitization of prostate cancer cells to Taxotere.** To test this prediction, we will:

2.1 determine whether the effect of DIM on cell proliferation and apoptosis in prostate cancer cells (LNCaP and C4-2B cells) correlates with our results on down-regulation of survivin associated with other genes, which could lead to chemo-sensitization of HRPC to Taxotere.
Please see the experimental design and methods for aim 1 above.
2.2 assess whether DIM in combination with Taxotere could enhance the inhibition of tumor growth *in vivo* using the SCID-hu animal model of experimental prostate cancer bone metastasis induced by LNCaP and C4-2B cells. We will also correlate *in vitro* and *in vivo* anti-tumor activity with the regulation of survivin and the expression of other genes in tumors treated with DIM and Taxotere.

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These results will be useful to establish molecularly based targeted therapy for the treatment of prostate cancer in general but most importantly for the treatment of HRPC and bone metastatic disease.

Previously we have used an animal model of experimental breast cancer bone metastasis that has been established in our laboratory supported by a small funding by DOD awarded to Dr. Rahman to test the chemopreventive as well as therapeutic effects of dietary I3C. Using this model, we have shown that NF-κB targeted genes could be down regulated by I3C. For these experiments, a similar SCID-hu xenograft model will be used to test whether DIM could sensitize prostate tumors to Taxotere. Suspensions of LNCaP and C4-2B prostate cancer cells ($1x10^5$ cells in a volume of 20 µl) will be injected into the implanted human fetal bone (Advance Bioscience Resources, Inc. Alameda, CA) by inserting 27g needle through the mouse skin directly into the bone marrow. Once the tumor is measurable, animals will be divided into four groups of 10 animals in each group. Group-I: untreated control; group-II: exposed to DIM (150 mg/kg body weight by gavage) or diet containing DIM (1000 ppm; this is based on previously published reports); group-III: treated with Taxotere alone (5 mg/kg body weight by i.v., every alternate day for three injections); group-IV: exposed to DIM and also treated with Taxotere as shown for groups-II and III. After 5 days following the treatment schedule, the animal tumors will be measured and data will be plotted. Mice from all experimental groups will be sacrificed five days after the end of all treatments, and tumors will be harvested from each animal and processed for preparation of nuclear proteins as well as tumor extracts for Western blot analysis for measuring the expression of AR, PSA, NF-κB and survivin following standard procedures with which the PI has extensive experience. Paraffin sections of the normal tissue and grown tumors collected from the experiments will be used for histologic and immunohistochemistry analysis.

Our progress was slow and we got an extension for one year with no cost. In fact we have been conducting a numerous experiments which are taking more time than anticipated previously. Hence, in order to demonstrate whether the chemo-enhancing effect of DIM is additive or synergistic, we need to do additional experiments and we will make progress on data analysis and manuscript writing during the extended year.

Although task 1 and 2 are in progress, however we are now reporting the research accomplishment associated with task 1 and 2 outlined in the statement of work:

Materials and Methods

Cell lines, reagents, and antibodies. Human prostate cancer cell lines including LNCaP and C4-2B were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 µg/mL of streptomycin in a 5% CO_2 atmosphere at 37°C. DIM was generously provided by Dr. Michael Zeligs (BioResponse) and was dissolved in DMSO to make a 50 mmol/L stock solution. Anti-PARP (BIOMOL, PA), anti-survivin (R&D system, MN), anti-caspase 3 (Cell signaling, MA), anti-Bax (Santa Cruz, CA), and anti-ß-actin (Sigma) primary antibodies were used for Western blot analysis. Taxotere (Aventis Pharmaceuticals, Bridgewater, NJ) was dissolved in DMSO to make a 4 µM stock solution.

Cell proliferation inhibition studies by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Human LNCaP and C4-2B prostate cancer cells were seeded in 96-well plates. After 24 hours, the cells were treated with 10, 30, and 40 µmol/L DIM followed by treatment of Taxotere with 0.5, 1.0 and 1.5 nM for 24 to 72 hours. Control cells were treated with 0.1% DMSO (vehicle control). After treatment, the cells were incubated with MTT (0.5 mg/mL, Sigma) in medium at 37°C for 2 hours and then with isopropanol at room temperature for 1 hour. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan, Durham, NC) at 595 nm.

Histone/DNA ELISA for detection of apoptosis. The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in prostate cancer cells treated with DIM according to the manufacturer's protocol. Briefly, the cytoplasmic histone/DNA fragments from LNCaP and C4-2B cells treated with 10, 30, and 40 µmol/L DIM followed by treatment of Taxotere with 0.5, 1.0 and 1.5 nM for 24 to 72 hours were extracted and incubated in microtiter plate modules coated with anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments, followed by color development with 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) substrate for peroxidase. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan) at 405 nm.

Western blot analysis. LNCaP and C4-2B cells were cultured in RPMI 1640 with 10% FBS or 10% dextran-coated charcoal-stripped FBS (DCC-FBS). Cells were then treated with DIM at various concentrations for different time periods followed by treatment with and without Taxotere for 72 hours. After treatment, cells were lysed and protein concentrations were then measured using bicinchoninic acid protein assay (Pierce, Rockford, IL). The proteins were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The membranes were incubated with various primary antibodies, and subsequently incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce).

NF-κB DNA-binding activity measurement. LNCaP and C4-2B cells were plated at a density of 1 x 10^6 in 100-mm dishes and cultured for 24 hours. Subsequently, the cultures were treated as described above. Nuclear extracts were prepared from control and DIM-treated breast epithelial cells as previously described (24, 73, 74) and subjected to analysis for NF-κB DNA-binding activity as measured by electrophoretic mobility shift assay. Using frozen tumor tissue, nuclear proteins were also extracted as described previously (24, 73, 74). Briefly, tissues were minced and incubated on ice for 30 minutes in 0.5 mL ice-cold buffer A composed of 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L DTT, 10% NP40, 0.1% IGEPAL CA-630, and 0.5 mmol/L phenylmethylsulfonyl fluoride. The minced tissue was homogenized using a Dounce homogenizer (Kontes Co., Vineland, NJ) followed by centrifugation at 5,000 x g at 4°C for 10 minutes. The supernatant (cytosolic proteins) was collected for Western blot analysis and kept at −70°C until use. The crude nuclear pellet was suspended in 200 μL buffer B [20 mmol/L HEPES (pH 7.9), 25% glycerol, 1.5 mmol/L MgCl₂, 420 mmol/L NaCl, 0.5 mmol/L DTT, 0.2 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, 4 μmol/L leupeptin] and incubated on ice for 30 minutes. The suspension was centrifuged at 16,000 x g at 4°C for 30 minutes. The supernatant (nuclear proteins) was collected and kept at −70°C until use. The protein concentration was determined using bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL). Electrophoretic mobility shift assay was done by preincubating 8.0 μg nuclear extract with a binding buffer containing 20% glycerol, 100 mmol/L MgCl₂, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl, and 0.25 mg/mL poly(deoxyinosinic-deoxycytidylic acid) for 10 minutes. After the addition of IRDye 700-labeled NF-κB oligonucleotide, samples were incubated for an additional 20 minutes. The DNA-protein complexes were electrophoresed in an

8.0% native polyacrylamide gel and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1.

Results

## A. DIM potentiated prostate cancer cell growth inhibition caused by Taxotere

LNCaP and C4-2B cancer cells were treated with DIM, Taxotere, or DIM in combination with lower doses of Taxotere. The cell viability was determined by MTT assay and the effect of DIM or Taxotere on the growth of different cancer cells is shown in Fig. 1A, 1B, 1C (LNCaP) and 1D, 1E, 1F (C42B) . We have tested several doses of DIM and Taxotere with different time points and we found that treatment of cells with DIM or Taxotere alone for 72 hours typically caused 40-70% growth inhibition in cancer cells using the doses tested (Fig. 1A,1B, 1D and 1E). However, DIM in combination with lower doses of Taxotere resulted in ~90% growth inhibition in both prostate cancer cells, suggesting the greater inhibitory effect of combination treatment (Fig. 1C and 1F). These results showed that combination of DIM along with lower doses of Taxotere elicited significantly greater inhibition of cancer cell growth compared with either agent alone. The lower dose of Taxotere in inhibiting cell growth when combined with a non-toxic agent (DIM) will have significant outcome for extending our studies for human prostate cancer treatment.

Figure 1:
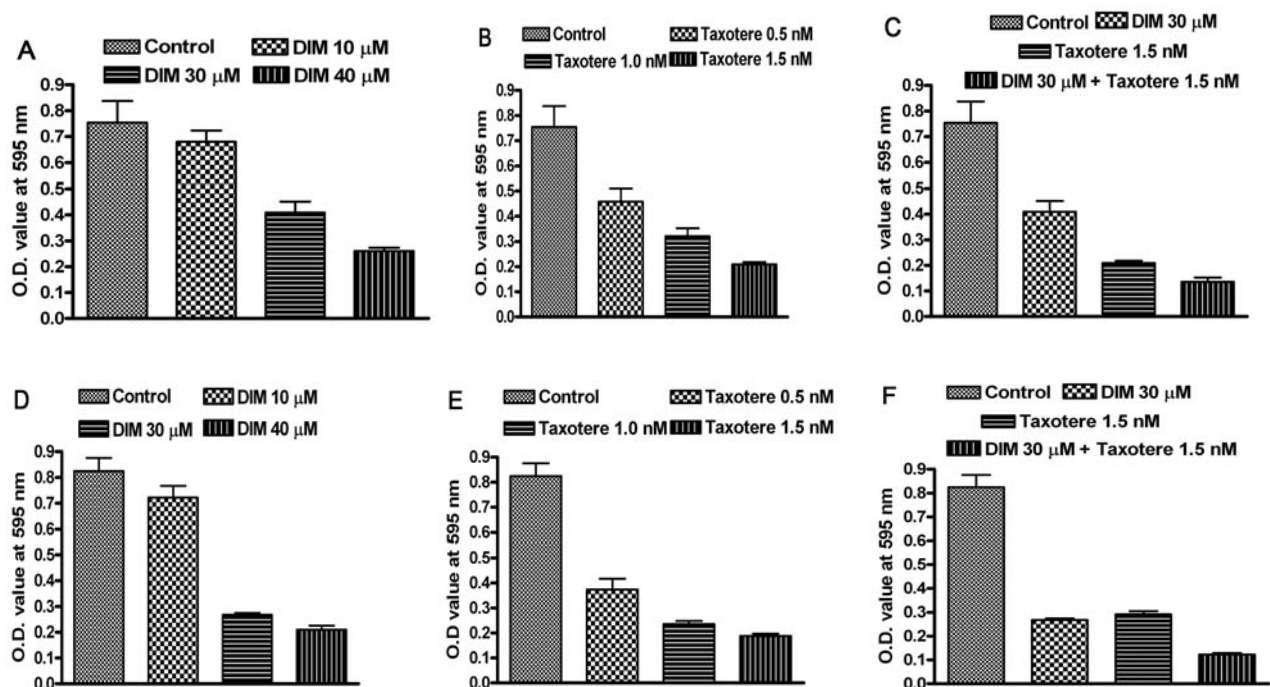
B. DIM sensitized prostate cancer cells to apoptosis induced by chemotherapeutic agents

Figure 2:
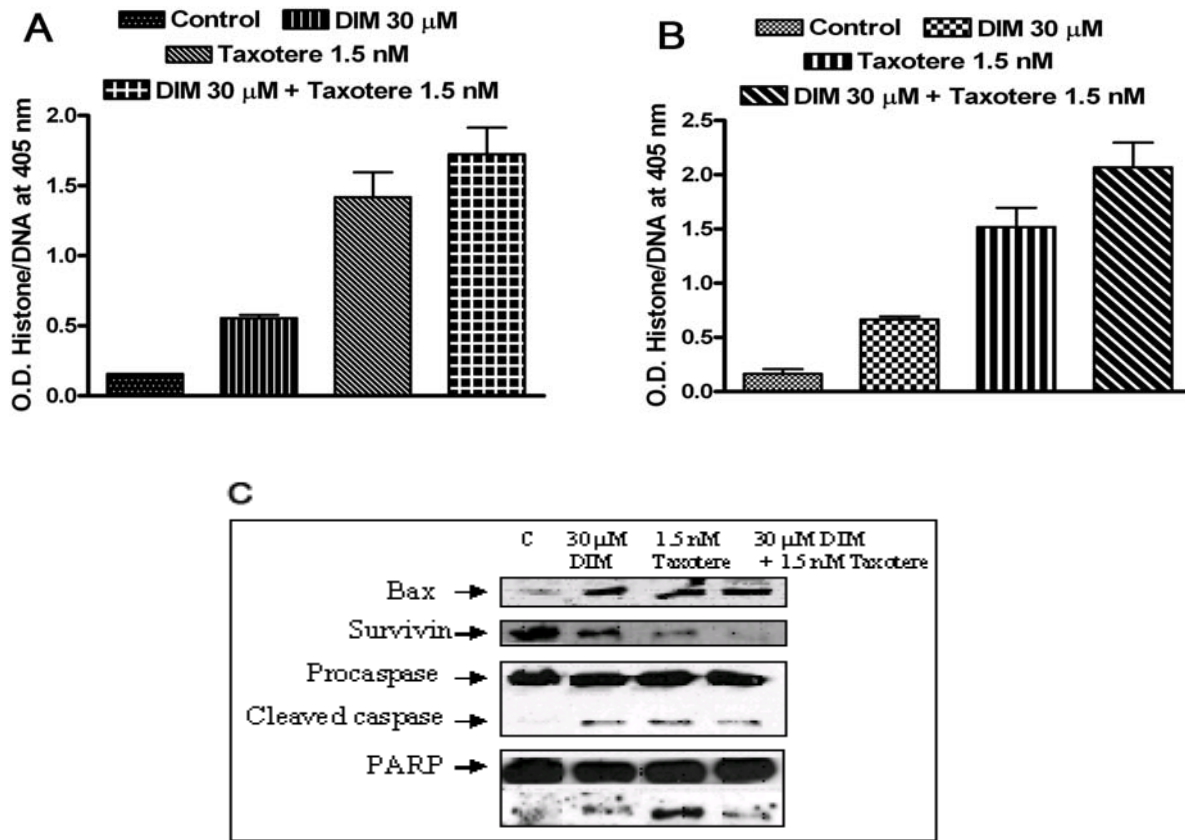
By apoptotic cell death ELISA we observed similar results showing that DIM or Taxotere induced apoptosis in prostate cancer cells tested (Fig. 2A, LNCaP cells and 2B, C42B cells). We found that 30 µM DIM combined with lower doses of Taxotere induced more apoptosis in the cancer cells compared with single-agent treatment (Fig. 2A and 2B). We also observed that DIM and Taxotere combination treatment produced more PARP cleavages and cleaved caspase compared with mono-treatment (Fig. 2C), suggesting more apoptosis induced by the combination treatment *in vitro*. By Western blot analysis, we found that DIM alone or in combination with chemotherapeutic agents down-regulated the expression of survivin and up-regulated Bax in LNCaP cells (Fig. 2 C). These results are consistent with cell growth inhibition observed by MTT assay, suggesting that greater cell growth inhibition by combination treatment may be mediated through the induction of more apoptosis in cancer cells.

C. DIM abrogated activation of nuclear factor κB (NF-κB) activity induced by Taxotere *in vitro*

Figure 3:
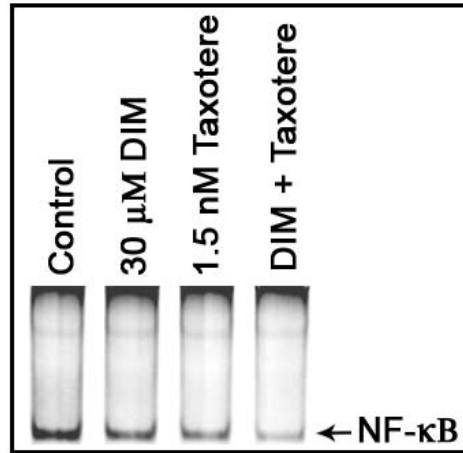
We next looked into molecular basis for chemo-resistance by performing EMSA in C4-2B prostate cancer cell using Taxotere and DIM. Nuclear proteins from cultured cancer cells treated with DIM and Taxotere were subjected to analysis for NF-κB DNA-binding activity as measured by EMSA. Our findings clearly showed that induction of NF-κB which can be down-regulated by combination treatment (Fig 3). These results provide mechanistic support in favor of our claim that the apoptosis-inducing effect of Taxotere is enhanced by DIM and it is partly mediated through the NF-κB pathway.

KEY RESEARCH ACCOMPLISHMENT

1. Our results showed that combination of DIM with lower doses of Taxotere elicited significantly greater inhibition of cancer cell growth compared with either agent alone.
2. Collectively our present results clearly suggest that inactivation of survivin mediated by the inhibition of NF-κB signaling by DIM leads to chemosensitization of prostate cancer cells to Taxotere which may contribute to increased growth inhibition and apoptosis in prostate cancer cells.

REPORTABLE OUTCOMES

1. KM Wahidur Rahman, Sanjeev Banerjee, Amir Ahmad and Zhiwei Wang (2008). DIM enhances Taxotere-induced apoptosis in hormone refractory prostate cancer cells through survivin down-regulation (in preparation).

CONCLUSIONS

In conclusion, out present results clearly show that DIM down-regulates survivin expression mediated by the inactivation of NF-κB and, in turn, sensitizes prostate cancer cells to growth inhibition and apoptosis induced by chemotherapeutic agents such as Taxotere. However, further in-depth studies including clinical trials are needed to fully evaluate the value of DIM in combination with chemotherapeutic agents such as Taxotere for the treatment of human cancers specially hormone refractory prostate cancer (HRPC).

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